

Regulation of alphavirus 26S mRNA transcription by replicase component nsP2

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Semliki Forest virus (SFV) mutant ts4 has a reversible temperature-sensitive defect in the synthesis of the subgenomic 26S mRNA. The viral nonstructural protein nsP2 was identified as a regulator of 26S synthesis by transferring nsP2 coding sequences from ts4 into the infectious SFV cDNA clone (SFots) to create SFots4. Sequencing identified the causal mutation as C4038U, predicting the amino acid change M781T in nsP2. A revertant was isolated in which a back mutation of U to C restored the wild-type phenotype. Compared to Sindbis virus nsP2 mutants ts15, ts17, ts18, ts24 and ts133, which also exhibit temperature-sensitive 26S RNA synthesis, ts4 and SFots4 reduced 26S RNA syn-

thesis faster and to lower levels after temperature shift. Under these conditions, ts4 and SFots4 also displayed complete conversion of RFII and RFIII into RFI and reactivated minus-strand synthesis. After shift to 39 °C, ts4 nsP2 was released from a crude RNA polymerase preparation consisting of membranes sedimenting at 15000 g (P15) and the remaining, unreleased nsP2 was capable of being cross-linked in almost equimolar ratio with nsP1 and nsP3. This supports the hypothesis that nsP2 binds directly or indirectly to the promoter for 26S RNA and that it is also an essential component of the viral replicase synthesizing 42S RNA plus strands. Only the former activity is temperature-sensitive in ts4 mutant.

Introduction

Semliki Forest virus (SFV) and Sindbis virus (SIN) belong to the alphavirus genus of the *Togaviridae* family of enveloped plus-stranded RNA viruses. The alphavirus genome consists of a single-stranded RNA of approximately 11.5 kb. Early in infection the genome RNA is translated as a large polyprotein (P1234) which, in SFV, consists of 2432 amino acid residues. The P1234 is autoproteolytically cleaved to non-structural proteins nsP1–nsP4, which are the virus-specific components of the cytoplasmic RNA polymerase (reviewed in Strauss & Strauss, 1994).

The parental genome is transcribed into complementary minus strands (SFV: 42S RNA[−]), which in turn are used initially as templates for the synthesis of new 42S RNA plus strands. These function again as templates for new 42S RNA minus strands. Thus, synthesis of both plus and minus strands

takes place during the first 1 to 4 h post-infection (p.i.) (exponential phase), whereafter the synthesis of minus strands is specifically shut off. Minus-strand synthesis needs ongoing protein synthesis (Sawicki & Sawicki, 1980). In contrast, stable viral RNA polymerases active even in the absence of protein synthesis use the minus-strand templates for continuous synthesis of 42S RNA plus strands and a subgenomic 26S RNA (reviewed in Strauss & Strauss, 1994).

The 26S RNA is identical in sequence to the 3'-third of the genome and codes for the structural proteins of the virus. Transcription of 26S RNA takes place from an internal promoter (Pettersson *et al.*, 1980). The minimal promoter consists of 19 nt upstream and 5 nt downstream (−19/+5 promoter) from the transcription start site (Levis *et al.*, 1990), whereas for optimal transcription a longer sequence is needed (−98/+14 nt; Raju & Huang, 1991).

The functions of the individual nsPs as polymerase components were studied using temperature-sensitive (ts) mutants. nsP4 is the catalytic subunit of the polymerase (Keränen & Kääriäinen, 1979; Barton *et al.*, 1988; Hahn *et al.*, 1989a), nsP1 is needed in the initiation of minus-strand synthesis (Sawicki *et al.*, 1981a; Wang *et al.*, 1991), and nsP2

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regulates 26S RNA synthesis and contains the viral non-structural protease activity (de Groot *et al.*, 1990; Strauss & Strauss, 1994). In contrast, the functions of nsP3 have remained undefined, although it was necessary for the RNA-positive phenotype of SIN (Hahn *et al.*, 1989b).

Some of the functions of the nsPs have been solved by expressing the individual proteins in bacteria and eukaryotic cells. Purified nsP2 has been shown to have NTPase activity (Rikonen *et al.*, 1994; Rikonen, 1996). Expression of nsP1 in *E. coli* and in insect cells showed that it has guanine-7-methyltransferase activity required in capping of mRNAs (Mi & Stollar, 1991; Laakkonen *et al.*, 1994; Wang *et al.*, 1996). NsP1 has also guanylyltransferase activity (Ahola & Kääriäinen, 1995; Ahola *et al.*, 1997). Furthermore, nsP1 is palmitoylated, which makes it strictly membrane associated (Laakkonen *et al.*, 1996). NsP3 of both SFV and SIN is a phosphoprotein (Peränen *et al.*, 1988; Li *et al.*, 1990).

We have previously characterized SFV temperature-sensitive RNA-negative mutant ts4 by temperature-shift experiments (Saraste *et al.*, 1977; Sawicki *et al.*, 1978). When the mutant was grown at permissive temperature (28 °C), both 42S genome RNA and 26S RNA were synthesized normally. When ts4-infected cultures were shifted to restrictive temperature (39 °C), the synthesis of 26S RNA ceased, while synthesis of 42S RNA continued. Synthesis of 26S RNA resumed when the cultures were shifted back to permissive temperature, indicating that the defect was temperature-dependent and reversible.

Here we have mapped the causative mutation of ts4 to the carboxy terminus of nsP2, where M781 has been changed into T. We compared SFV ts4 with several SIN mutants with defects in 26S synthesis. We show that this change in ts4 nsP2 at 39 °C is responsible for (i) inhibition of P1234 processing, (ii) inhibition of normal shutoff of minus-strand synthesis, and (iii) shutoff of 26S synthesis, possibly by detaching from the subgenomic promoter.

Methods

Cell cultures and viruses. The cultivation of baby hamster kidney cells (BHK21) was as described by Sawicki *et al.* (1981a). The origin and propagation of cloned SFV wild-type and ts4 mutant (Keränen & Kääriäinen, 1974), and SIN HR and group A ts mutants have been described previously (Sawicki & Sawicki, 1985). SFoto, used as control virus for recombinant and revertant viruses, was derived from infectious clone pSP6-SFV4 (a gift from H. Garoff and P. Liljeström, Karolinska Institutet, Stockholm, Sweden) by transfection of transcribed RNA into BHK cells (Liljeström *et al.*, 1991), and was renamed SFoto to distinguish it from SFV wt, the parent of ts4. Recombinant viruses SFots4SB and SFots4 were derived from infectious cDNA clones as described below. To obtain SFots4, individual plaques were picked and the second passage was used in further experiments. SFots4R, a revertant of SFots4, was grown at 37 °C after two sequential plaque purifications at 39 °C.

Construction of recombinant plasmids and sequencing. DNA manipulations and cloning were performed using standard methods

(Sambrook *et al.*, 1989). The cDNA of the original ts4 was digested with *SacI* and *BglII* restriction enzymes (nt 1802–6712), comprising 2288 nt of the nsP2 gene (excluding the first 105 nt), the whole nsP3 gene and 1172 nt from the beginning of the nsP4 gene. The resulting fragment was swapped in place of the parental restriction enzyme fragment in the infectious clone, pSFoto, to create pSFots4SB, which was subcloned further. The *SacI*–*BglII* fragment of pSFoto was cloned first into the pGEM3 vector, which had been digested with the same enzymes. Then a *PvuII* fragment (nt 2923–4094) of pSFots4SB was cloned into pGEM3–*SacI*–*BglII* (SFoto), replacing the parental *PvuII*–*PvuII* fragment. From plasmid pGEM3–*SacI*–*PvuII*(ts4)–*BglII* the *SacI*–*BglII* piece, which encoded amino acids 390–799 of ts4 nsP2, was recloned into pSFoto cDNA to get pSFots4 (Fig. 1a). The nsP2 gene was sequenced by the dideoxy-chain termination method using a commercial T7 sequencing kit (Pharmacia). SP6 and T7 primers were from Promega Biotech; the other oligomers for sequencing were from the Institute of Biotechnology, University of Helsinki and from OPERON.

Labelling of RNAs and isolation of replicative forms (RFs). BHK cells in 35 mm plastic petri dishes (about 2×10^6 cells) were infected at 28 °C with 100 p.f.u. per cell and labelled with 50 μ Ci [3 H]uridine/ml (30 Ci/mmol; NEN) in the presence of actinomycin D (2 μ g/ml) for the times indicated in the text. The RNAs were isolated, and the acid insoluble radioactivity was determined (Dé *et al.*, 1996). The 42S and 26S RNAs (usually from 1×10^6 cells) were separated by electrophoresis on 0.8% agarose gels (Wang *et al.*, 1994) or in 15–30% sucrose gradients (Keränen & Kääriäinen, 1974). Incorporation of [3 H]uridine into each RNA was quantified from dried gels after autoradiography by counting the radioactivity in the bands, or by laser densitometry of exposed films (Laakkonen *et al.*, 1994). The rate of inhibition of 26S synthesis was determined after shifting the infected cells from 28 °C to 39 °C at 5 or 7 h p.i. by labelling with 100 μ Ci [3 H]uridine per 2×10^6 cells for 15 min.

The double-stranded replicative forms (RFI, II, III) were isolated from the viral replicative intermediates (RIs) from infected cell extracts, pulse labelled at 39 °C for 15 min periods, and harvested at 45, 60, 120, 180 and 240 min after shift up to 39 °C (aliquots of 2×10^6 cells), as described previously (Wang *et al.*, 1994). For determination of minus-strand synthesis, purified RFs were obtained by RNase A treatment of the [3 H]uridine-labelled viral RIs and subsequent chromatography of the RFs on CF-11 cellulose (Sawicki & Sawicki, 1986). The RF RNA was heat-denatured and hybridized in the presence of an excess of unlabelled, purified 42S virion plus-strand RNA (Sawicki & Sawicki, 1986; Wang *et al.*, 1994).

Labelling of nsPs and analysis by immunoprecipitation. Infected BHK cells incubated at 28 °C were labelled for 30 min with 50 μ Ci [35 S]methionine/ml (1000 Ci/mmol, ICN Radiochemicals) at 4 h p.i. after treatment with hypertonic medium (Sawicki & Gomatos, 1978), followed by chase periods of 15 and 60 min. Labelled proteins were separated by SDS-PAGE in 6% gel according to Laemmli (1970), and visualized by fluorography (Laakkonen *et al.*, 1994). For immunoprecipitation, infected BHK cells in 100 mm dishes were labelled at 28 °C from 2 to 4 h p.i., followed by chase in the presence of cycloheximide (100 μ g/ml) from 4 to 8 h p.i. Duplicate cultures were shifted to 39 °C at 6 h p.i., and half of them were returned to 28 °C after 1 h at 39 °C. Cells were harvested at 8 h p.i. and the postnuclear supernatant was centrifuged at 15 000 g for 20 min to obtain pellet (P15) and supernatant (S15) fractions. The immunoprecipitations were carried out after denaturation of the proteins in 1% SDS (Laakkonen *et al.*, 1994), followed by SDS-PAGE (in 7.5% minigels) and fluorography. Incorporation in individual protein bands was quantified by using a Shimadzu scanner. For cross-linking of labelled nsPs, 450 μ l of S15 and P15 fractions were

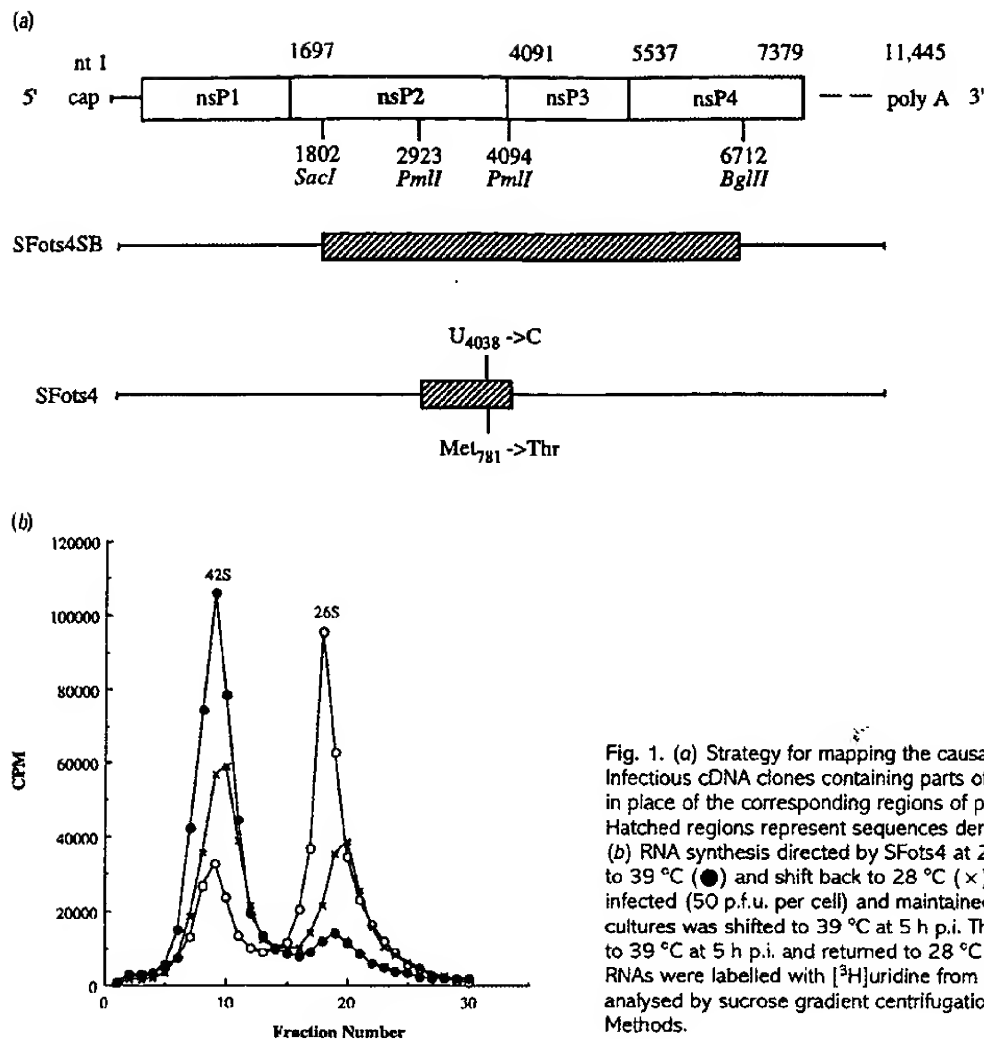


Fig. 1. (a) Strategy for mapping the causal mutation in SFV ts4. Infectious cDNA clones containing parts of ts4 nsP coding region in place of the corresponding regions of parental pSFoto cDNA. Hatched regions represent sequences derived from ts4 mutant. (b) RNA synthesis directed by SFots4 at 28 °C (○), after shift to 39 °C (●) and shift back to 28 °C (×). BHK cultures were infected (50 p.f.u. per cell) and maintained at 28 °C. One set of cultures was shifted to 39 °C at 5 h p.i. The other set was shifted to 39 °C at 5 h p.i. and returned to 28 °C at 7 h p.i. The viral RNAs were labelled with [³H]uridine from 8–9 h p.i. and the RNAs analysed by sucrose gradient centrifugation as described in Methods.

incubated with 0.1 mg/ml DTSP (dithiobis-succinylpropionate, 10 mg/ml in DMSO; Sigma) for 20 min on ice. Reaction was stopped by adding 0.2 M glycine (1:10) and incubating for 10 min at room temperature. Denatured 100 µl aliquots were immunoprecipitated with anti-nsP1-nsP4 antisera, followed by SDS-PAGE in 15 % acrylamide gels and fluorography.

■ **In vitro RNA polymerase assay.** P15 membranes from SFoto- and SFots4-infected BHK cells in 150 mm petri dishes (about 65×10^6 cells per dish) were isolated and the RNA polymerase activity and product analysis were carried out as described previously (Barton *et al.*, 1988).

Results

The causal mutation in SFV ts4 maps to nsP2

To map the ts4 lesion, most of the nsP coding region of ts4 was first swapped in place of the parental sequence in the SFV infectious clone pSP6-SFV4, which was renamed pSFoto, (virus: SFoto) to yield recombinant plasmid pSFots4SB (Fig. 1a). SFots4SB was temperature-sensitive for growth (Table 1). The insert was further subcloned as described in Methods. The resulting SFots4 was phenotypically similar to ts4 (Table 1). Sequencing of the insert and comparison with its wild-type

parent revealed only one nucleotide change, U4038C, which altered ATG codon to ACG, i.e. from M781 to T (Fig. 1a). We isolated a revertant (SFots4R), which had a wild-type phenotype (e.o.p. 10^{-1}). Sequencing of the *PmlI*–*PmlI* fragment (nt 2923–4094) revealed only a back mutation of C4038 to U (Fig. 1a, Table 1), confirming that the single amino acid change was responsible for the ts4 RNA-negative phenotype.

A single mutation M781T has multiple effects

In order to show that this mutation was responsible for the ts4 phenotype, the processing of P1234 and synthesis of 26S RNA in SFots4-infected cells were studied. As shown previously for ts4 (Sawicki & Gomatos, 1978), polypeptide precursors P1234, P123, P12 and P34 were slowly processed when SFots4-infected were shifted from 28 °C to 39 °C, whereas cleavage at 28 °C was similar to SFoto. In the revertant SFots4R-infected cells P1234 was cleaved rapidly also at 39 °C (data not shown). Thus, the revertant virus showed full recovery of the protease activity. RNA synthesis was studied by shifting the infected cells to 39 °C after 6 h

Table 1. Analysis of original and recombinant viruses

Virus	Fragment replaced (nt)	e.o.p.*	Temperature-sensitive		
			Virus growth	RNA synthesis	Protease
SFots		3.0×10^{-1}	—	—	—
wt		2.0×10^{-1}	—	—	—
ts4		0.6×10^{-6}	+	+	+
SFots4SB	1802–6712	ND	+	+	ND
SFots4	2923–4094	1.8×10^{-6}	+	+	+
ts4R		2.3×10^{-1}	—	—	—
SFots4R	2923–4094	3.6×10^{-1}	—	—	—

* Efficiency of plaque formation (p.f.u. at 39 °C/p.f.u. at 28 °C).

ND, Not determined.

incubation at 28 °C, followed by labelling of the viral RNAs with [3 H]uridine in the presence of actinomycin D. The reversibility of the defect was tested by transferring part of the infected cultures back to 28 °C before labelling. In this experiment the 42S and 26S RNAs were separated by sedimentation in 15–30% sucrose gradients (Fig. 1*b*). SFots4 reproduced the pattern of 26S synthesis, previously described for SFV ts4 (Sawicki *et al.*, 1978). Synthesis of 26S RNA was inhibited at 39 °C, while the synthesis of 42S RNA increased, leading to a significant increase in the molar ratio of 42S RNA:26S RNA. When SFots4-infected cells were transferred back to 28 °C after 2 h at 39 °C, the synthesis of 26S RNA recovered (Fig. 1*b*).

Comparison of SFV and SIN nsP2 mutants in temperature-shift experiments

The RNA-negative nsP2 mutants SIN ts15, ts17, ts24 and ts133, assigned to complementation group A1, and SIN ts18 (group G) (Sawicki & Sawicki, 1985) were studied together with SFV ts4. The shutoff of 26S synthesis was most prominent and rapid with SFots4, which reached molar ratios of 5.2 3 h after shift to 39 °C (Fig. 2). Taking the amount of 26S RNA synthesized at 28 °C prior to the shift up as 100%, 26S synthesis decreased progressively to less than 10% after 3 h at 39 °C with both ts4 and SFots4. A much slower decrease of 26S synthesis was seen with SIN mutants ts18 and ts133 (Fig. 2). Overall, the results suggest that the SIN nsP2 mutants lost 26S synthesis somewhat more slowly and less completely than SFots4 after shift to 39 °C.

Reversibility of SFots4 26S synthesis was studied by shifting the infected cultures first from 28 °C to 39 °C for 1–3 h, followed by shift back to 28 °C, and labelling with [3 H]uridine at hourly intervals. The shift to 39 °C was carried out at 5 h and 7 h p.i. Reversion of 26S synthesis took place within the first hour after shift to 28 °C, independent of the time at 39 °C (Fig. 3*a*). Similar results were obtained when protein synthesis was inhibited with cycloheximide at the time of shift to 39 °C (data not shown). Changes in the molar ratios

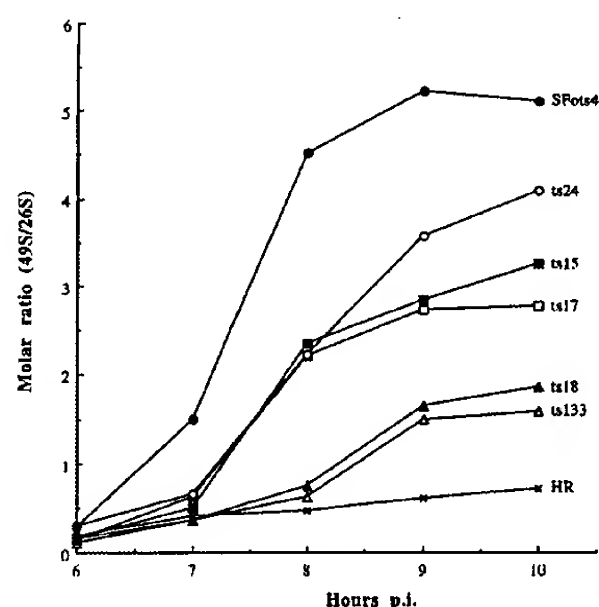


Fig. 2. Molar ratios 42S:26S RNA of SFots4 and Sindbis group A mutant virus-infected cells. BHK cultures were infected (100 p.f.u. per cell) at 28 °C with SFots4, SIN HR and mutants ts15, ts17, ts18, ts24 and ts133. At 5 h p.i. they were transferred to 39 °C. The continued subgenomic and viral RNAs were pulse-labelled starting from 45 min after shift up. 42S and 26S RNAs were separated by agarose gel electrophoresis and quantified as described in Methods. The results shown represent average of four (SIN viruses) and five (SFots4) experiments.

were less extensive when the shift to 39 °C was carried out 7 h p.i. (Fig. 3*a*). Reversion of the SIN ts mutants was also rapid, essentially taking place 1 h after shift to 28 °C (Fig. 3*b*).

We wanted to study whether the reversion of 26S synthesis of SFots4 could take place also *in vitro*. The P15 (membrane pellet) fraction was isolated from cultures shifted to 39 °C at 7 h p.i. *In vitro* RNA synthesis was carried out at 28 °C for 30 min followed by isolation and analysis of the RNAs. With SFots4, there was only a partial recovery of 26S synthesis in P15 preparations isolated after 1 h or 2 h incubation at 39 °C. Essentially no 26S RNA was synthesized in preparations derived from cultures kept at 39 °C for 3 h (Fig. 3*c*, lane 8).

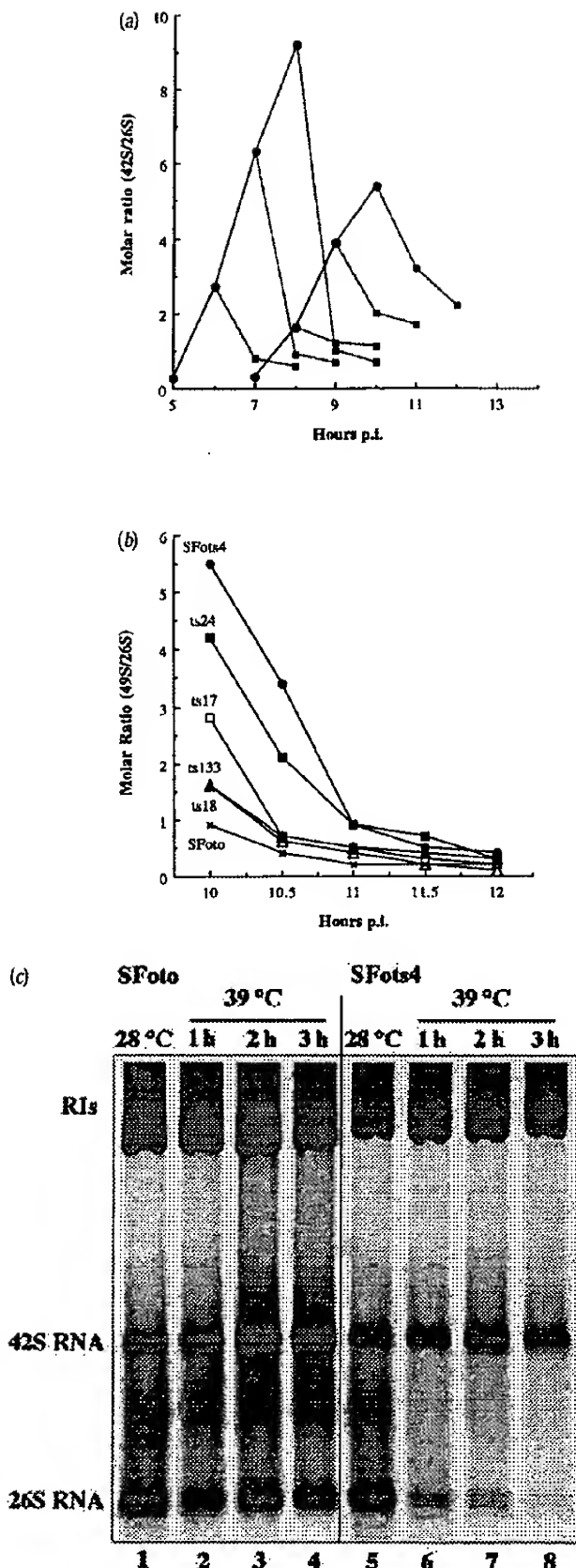


Fig. 3. Recovery of 26S RNA synthesis *in vivo* (a, b) and *in vitro* (c). (a) BHK cells infected with SFots4 were incubated at 28 °C for 5 h or 7 h after infection followed by shift to 39 °C (●), and returned to 28 °C after

Conversion of RFII/RFIII to RFI and loss of 26S mRNA synthesis

When viral RNA RIs are treated with small amounts of RNase A, three double-stranded RNAs (RFI, RFII and RFIII) are released. RFI represents a duplex of 42S RNA and is considered to be derived from replicative intermediate RI_n , which synthesizes 42S RNA plus strands. RFIII is a duplex of 26S RNA, which together with RFII (representing the duplex of the 5'-two-thirds of the 42S RNA) are derived from RI_b (Simmons & Strauss, 1972). RI_b contains a 42S minus-strand template, the 5'-third of which is actively transcribed to 26S RNA. The 26S RNA promoter area is extremely sensitive to nucleases after extraction to obtain protein-free RNA. It is assumed that the sensitivity is a result of the removal of a regulatory protein, which is bound to the promoter on the minus strand (Kääriäinen & Söderlund, 1978; Grakoui *et al.*, 1989).

We labelled the RIs by 15 min pulses with [3 H]uridine and isolated the RFs by agarose gel electrophoresis at different times after shift to 39 °C (Fig. 4a). The relative amount of labelled RFI, RFII and RFIII was determined by cutting and counting the individual RNA bands or by laser densitometry. Similar analysis was carried out for SIN mutants ts15, ts17, ts18 and ts24. No labelling of RFIII could be seen in SFots4-infected cells 3 h after shift of the cultures to 39 °C. In contrast, in SIN ts17- and ts24-infected cells (Fig. 4b, lane 5) there was only a 30–60% decrease of labelled RFIII during a 4 h incubation at 39 °C. Similar results were obtained for SIN ts18 and ts15 (data not shown).

Regulatory role of nsP2 in the synthesis of 26S RNA

Both ts4 and SFots4 showed a complete interconversion of RI_b to RI_n , as judged by the disappearance of RFIII and RFII. This suggested that the hypothetical regulatory protein or 'initiation factor' for 26S RNA synthesis had detached completely from the minus-strand template after shift of the cultures to 39 °C. Since the causative lesion was in nsP2 of ts4, we studied the distribution of it and other nsPs using crude cell fractionation. To this end proteins were labelled with [35 S]-methionine from 2–4 h p.i. at a time when new replication complexes are being formed. The cultures were chased from 4–8 h p.i. in the presence of cycloheximide to prevent the synthesis of uncleaved nsP precursors at 39 °C. Duplicate

1, 2 or 3 h incubation at 39 °C (■); (b) Cultures infected with SFots4 or SIN mutants ts17, ts18, ts24 and ts133 were incubated at 28 °C for 7 h, shifted to 39 °C for 3 h and then returned to 28 °C. Viral RNAs were labelled by 30 min pulses of [3 H]uridine (50 μ Ci per 2×10^5 cells) at the indicated times in (a) and (b). (c) For *in vitro* assay P15 membranes of SFoto (lanes 1–4) and SFots4 (lanes 5–8) were isolated 1, 2 or 3 h after shift to 39 °C at 7 h p.i. The recovery of 26S synthesis in P15 membranes was measured by carrying out *in vitro* RNA synthesis for 30 min at 28 °C using [35 S]CTP as label. Labelled viral RNAs were separated by electrophoresis on 0.8% agarose-TBE gels, the 42S and 26S RNA bands were cut, and the radioactivity was determined by liquid scintillation counting.

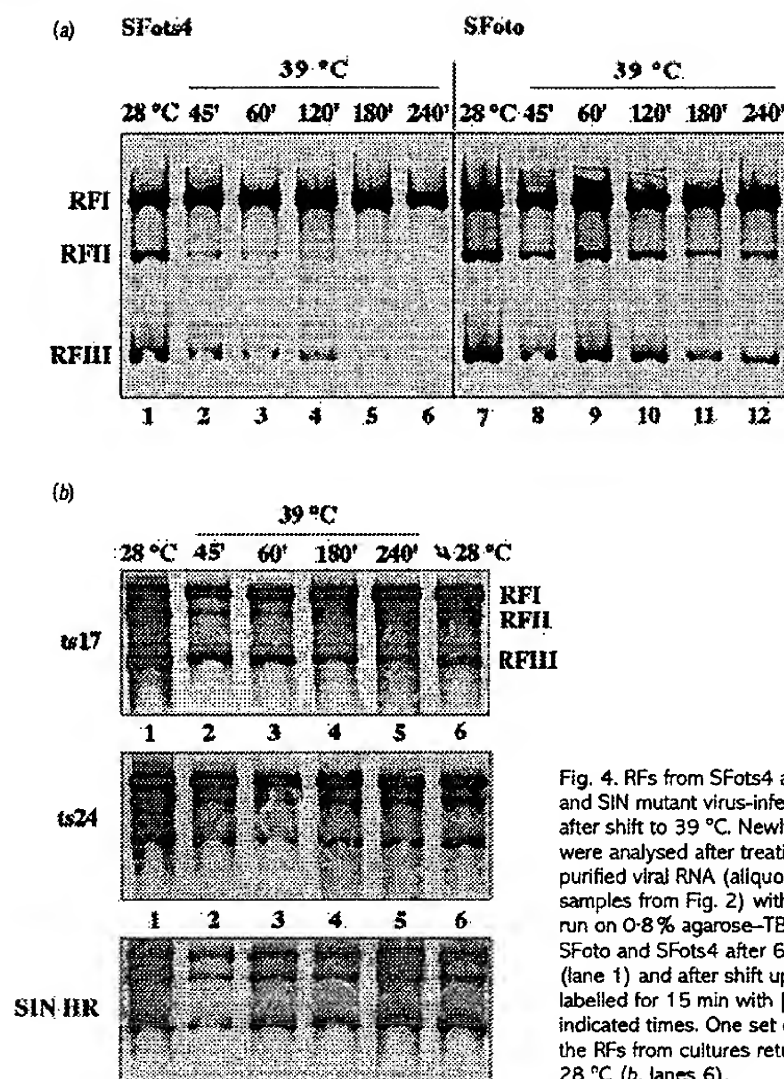


Fig. 4. RFs from SFots4 and SFoto (a) and SIN mutant virus-infected cells (b) after shift to 39 °C. Newly labelled RFs were analysed after treating isolated and purified viral RNA (aliquots of SIN samples from Fig. 2) with RNase A and run on 0.8% agarose-TBE gel. (a) RFs of SFoto and SFots4 after 6 h p.i. at 28 °C (lane 1) and after shift up at 5 h p.i., labelled for 15 min with [³H]uridine at indicated times. One set of samples show the RFs from cultures returned back to 28 °C (b, lanes 6).

cultures were shifted to 39 °C at 6 h p.i. The S15 and P15 fractions were isolated at 8 h p.i., and the distribution of the nsPs was determined by immunoprecipitation with mono-specific antibodies. The relative proportion of each nsP in the P15 and S15 fractions was quantified by scanning fluorographs of each gel.

The only consistent and significant, temperature-dependent difference was in the nsP2 distribution of ts4 (Fig. 5a, lanes 6 and 8). Approximately 70% of all immunoprecipitated cytoplasmic nsP2 was found in the S15 fraction 2 h after shift to 39 °C. When the relative amounts of nsP2 (in arbitrary A units) present in each fraction at 39 °C were compared with those at 28 °C, there was about a twofold decrease in the levels of nsP2 in P15 fractions. The average 39 °C/28 °C ratio of nsP2 in P15 fractions was 0.72 for SFV wt/SFoto and 0.33 for ts4/SFots4 (average of four experiments). The reversibility of nsP2 distribution was studied by shifting infected cultures first to 39 °C for 1 h (Fig. 5b, lanes 1–2) followed by shift back to 28 °C for 1 h (Fig. 5b, lanes 3–4). In SFots4-infected cells, the

amount of nsP2 in P15 fractions was restored to the wild-type level, showing that nsP2 was reversibly associated with the P15 fractions.

We used also the reversible cross-linking agent DTSP to treat ts4-infected cells which had been labelled with [³⁵S]-methionine as above. Cross-linking was carried out in P15 and S15 fractions derived from cultures incubated at 28 °C or shifted to 39 °C. Fig. 5(c) shows immunoprecipitations from ts4- and wt SFV-infected cells. In the S15 fraction, only nsP2 was precipitated by anti-nsP2 at both temperatures (Fig. 5c, lanes 1 and 3). In the P15 fraction from wt- and ts4-infected cells incubated at 28 °C and from wt-infected cells at 39 °C, nsP2 was cross-linked with nsP3 and nsP1 (Fig. 5c, lanes 2 and 4), indicating close proximity of nsP2 with these polymerase components. However, from its greater amounts, most of the precipitated nsP2 was evidently associated with the membrane fraction without contact with other nsPs. The situation was clearly different in ts4-infected cells shifted to 39 °C, since nsP2, nsP1 and nsP3 were found in close to equimolar amounts

(lane 4). We propose that the 'free nsP2' at 28 °C was associated with the 26S promoter, whereas the cross-linked nsP2 represented a component of the polymerase complex.

ts4 mutation affects synthesis of 42S RNA minus strands

Normally, minus strands are synthesized only early in infection, and their synthesis ceases 3–4 h and 5–6 h p.i. at 39 °C and 28 °C, respectively (Sawicki *et al.*, 1981a, b). The kinetics of minus-strand synthesis of SFoto and SFots4 were of interest because SIN mutants ts17 and ts133 have been shown to reactivate minus-strand synthesis in the absence of protein synthesis when the cultures are transferred to 39 °C (Sawicki & Sawicki, 1985, 1993). SFots4 differed from the SIN mutants since it did not shut off minus-strand synthesis even at 28 °C (Fig. 6). However, a further increase of minus-strand synthesis was repeatedly observed in cultures shifted to 39 °C in the presence of cycloheximide (Fig. 6), consistent with reactivation of ts4 minus-strand synthesis at 39 °C.

Discussion

Genetic studies, summarized in Fig. 7, have revealed that (i) nsP2, and in particular its carboxy-terminal half, plays an important role in the regulation of 26S mRNA synthesis (Keränen & Kääriäinen, 1979; Sawicki & Sawicki, 1985). (ii) Many of the SIN nsP2 mutants with defective 26S RNA synthesis had also a defect in the processing of the non-structural polyprotein (P1234), suggesting a defective proteinase function (Keränen & Kääriäinen, 1979; Sawicki & Sawicki, 1985). Recently it has been shown that the carboxy-terminal part of nsP2 also is a papain-like protease, which autocatalytically cleaves P1234 both *in vivo* and *in vitro* (Ding & Schlesinger, 1989; Strauss *et al.*, 1992; see also Strauss & Strauss, 1994). (iii) SIN conditionally lethal nsP2 mutants ts17 and ts133 can reactivate minus-strand synthesis in the absence of protein synthesis after shift to 39 °C (Sawicki & Sawicki, 1993). (iv) Unlike subgroup AI mutants that map to the carboxy-terminal half of nsP2, SIN subgroup AII mutants of nsP2 (ts14, ts16 and ts19) map to the amino-terminal part of nsP2 and show neither ts protease nor 26S RNA synthesis defects (Dé *et al.*, 1996).

In this and previous studies, SFV ts4 has been shown to have defects similar to those of SIN subgroup AI mutants (Fig. 7), i.e. slow cleavage of P1234 and shutoff of 26S RNA synthesis after shift to 39 °C. In this study, the protease defect was eliminated by allowing the synthesis and cleavage of the RNA polymerase components at 28 °C before shift to 39 °C. Thereafter we followed the shutoff of 26S RNA synthesis as a function of time and conversion of RFs. The SIN group AI mutants were used as controls. It turned out that ts4 (and SFots4) was most effective in the cessation of 26S RNA

synthesis after shift to 39 °C. Our comparison of the loss of 26S synthesis by SFV and SIN mutants found that inhibition was progressive and increased with time at 39 °C, differentiating this process from the very rapid loss of elongation by mutant nsP4 encoded by SIN ts6 (this study; Barton *et al.*, 1988). These results also confirm the earlier report by Scheele & Pfefferkorn (1969), who found loss of 26S synthesis by SIN ts24 at 39 °C was progressive and not immediate, and extend their single finding to a general observation affecting all 26S-defective mutants. Progressive loss and its more rapid recovery are intriguing aspects of the internal initiation process used by alphaviruses and are not fully understood.

The conversion of RFIII + RFII to RFI in SFV ts4-infected cells was complete after 3 h incubation at 39 °C, suggesting a quantitative detachment of the putative regulatory protein from the 26S RNA promoter in the 42S minus strand. Since the causative lesion in ts4 was in nsP2, we assumed that this protein was the predicted regulator. To study this possibility we labelled the nsPs at 28 °C early in infection and determined their distribution after shift to 39 °C in the P15 fraction, which contains essentially all the virus-specific RNA polymerase activity (Ranki *et al.*, 1979). If nsP2 detached from the template RNA, it would be released into the supernatant (S15), whereas the other nsPs would be retained in the P15 fraction. We found some nsP2 was released and some always remained in association with the other nsPs. Cross-linking of nsPs in the P15 fraction from ts4-infected cultures at 39 °C showed roughly equimolar ratios of nsP2 with nsP1 and nsP3, further supporting the idea that only a fraction of nsP2 is associated with the RNA polymerase complex (Fig. 5c), where it functions presumably as NTPase and RNA helicase (Rikonen *et al.*, 1994). The released nsP2 had the expected properties of the 26S 'regulator'.

Restoration of 26S synthesis at 28 °C in ts4-infected cells was rapid, suggesting that nsP2 reassociated with the 26S promoter quickly. However, restoration *in vitro* at 28 °C was poor in P15 fractions isolated from ts4-infected cells incubated at 39 °C. We assume that RNA synthesis takes place within closed membrane vesicles. When nsP2 is detached from the promoter it remains in the closed space and can reattach immediately after shift to 28 °C. Once the cells were broken, the membrane vesicles became leaky, nsP2 escaped and, after centrifugation, was found in the S15 fraction. Since nsP2 leaked out during P15 preparations, no restoration of 26S synthesis could occur during *in vitro* incubation at 28 °C. Conversion of RFs II and III to RFI in SIN nsP2 mutants was clearly less complete, although 26S RNA synthesis was shut off efficiently at 39 °C, and reverted rapidly after cultures were returned to 28 °C (Fig. 4b). We suggest that the shutoff of 26S synthesis by the SIN mutants was due to inactivation of nsP2 proteins that remained attached to the promoter (*in situ* inactivation).

The viral nsPs assemble into replicases active in 42S RNA plus and RNA minus strand synthesis and into transcriptases active in 26S RNA synthesis. Genetic and biochemical analyses

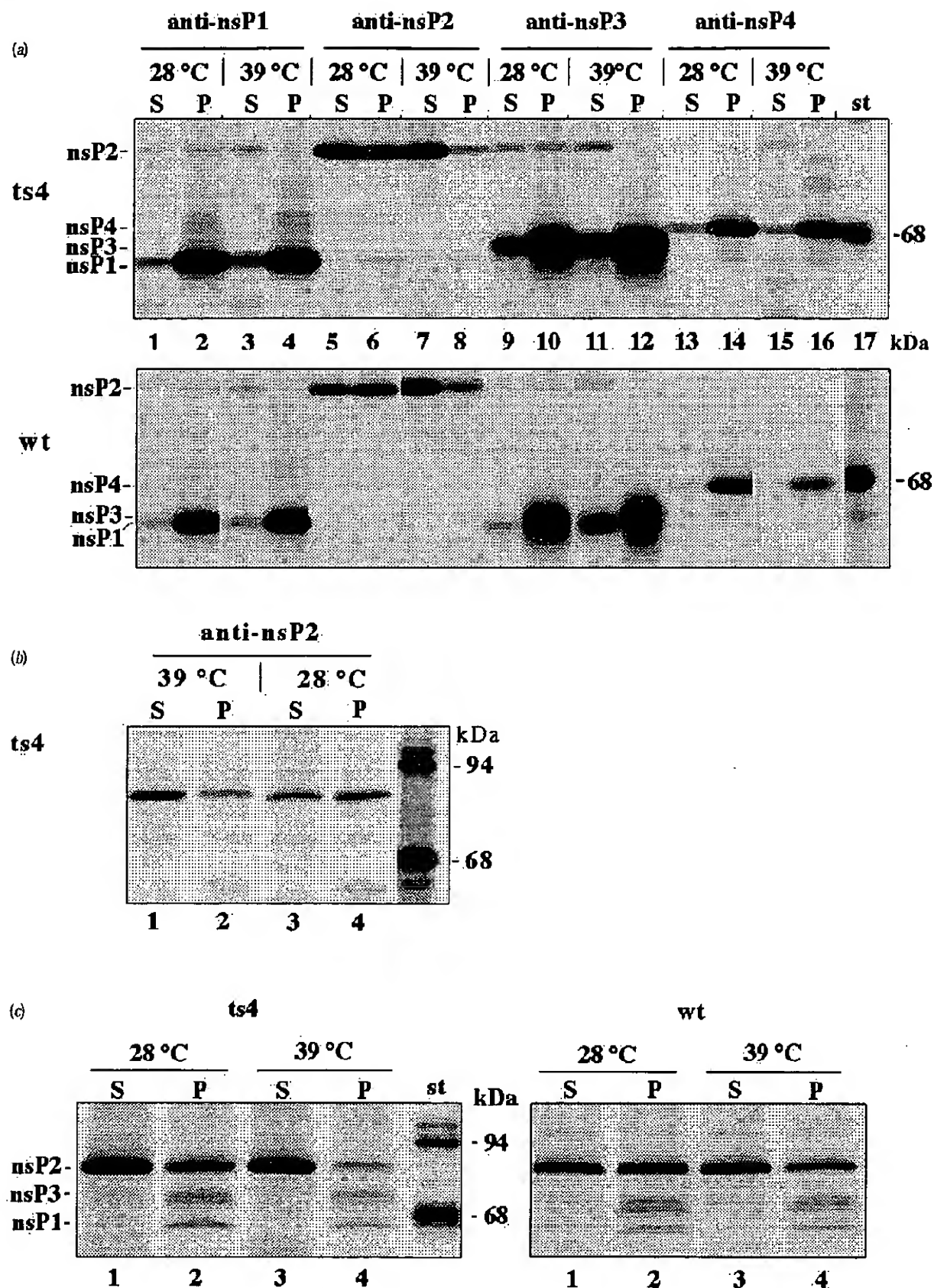


Fig. 5. (a) Temperature-dependent distribution of SFV ts4-specific non-structural proteins. BHK cells infected with ts4 or wild-type virus (wt) at 28 °C were labelled with [³⁵S]methionine between 2 and 4 h p.i. followed by a chase in the presence of cycloheximide (100 µg/ml). Half of the cultures were maintained at 28 °C (lanes 1, 2, 5, 6, 9, 10, 13 and 14) and half were

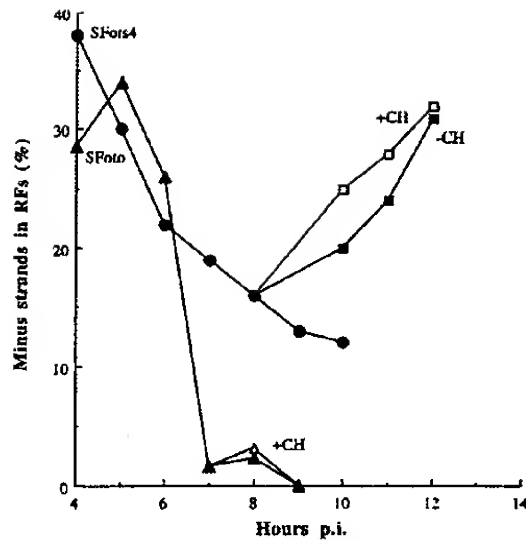
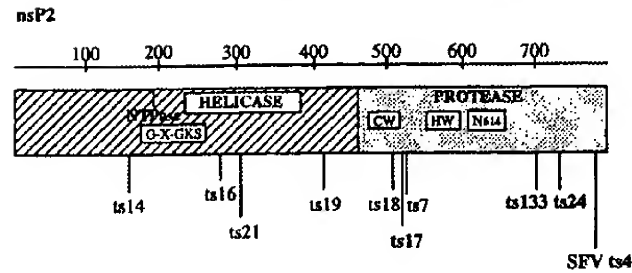


Fig. 6. SFots4-directed minus-strand RNA synthesis. Incorporation of [³H]uridine into purified RF RNAs from SFots4- and SFoto-infected cells incubated at 28 °C continuously or shifted to 39 °C at 8 h p.i. in the presence (+ CH) and absence (–CH) of cycloheximide. Results show average percentage of minus strands in isolated RFs. SFoto (▲) two experiments; SFoto + CH (△); SFots4 at 28 °C (●) six experiments shifted to 39 °C (+CH, □) (–CH, ■).

indicate that while minus-strand replicases require nsP2–nsP3 polyprotein forms (P123 or P23) for activity and plus-strand replicases are nsP1 + nsP23 + nsP4 or fully cleaved nsP complexes, only the fully cleaved nsP1 + nsP2 + nsP3 + nsP4 complexes are functional in 26S synthesis (Lemm *et al.*, 1994; Shirako & Strauss, 1994; Wang *et al.*, 1994). Thus, cleavage at the 2/3 site to release nsP2 and nsP3 as separate polypeptides is essential for recognition of the internal promoter on the minus strand and initiation of 26S synthesis. This event in turn would inactivate P23 and shut down minus-strand synthesis by the complex. The need for specific processing steps in addition to those needed to form the 42S RNA plus strand replicase support this and previous studies of alphavirus transcription, which demonstrated the synthesis of 26S mRNA was regulated independently of genome synthesis.

The third defect was reactivation of minus-strand synthesis after shift to 39 °C in the absence of protein synthesis, similar to that described for SIN ts17 and ts133. Substitution in nsP2 of SIN ts7 can also reactivate minus-strand synthesis after shift to 39 °C, but this mutation is not conditionally lethal (Wang *et al.*, 1994). The continuous production of minus strands seems



Mutant	Amino acid change	Temperature sensitive			
		Growth	26S RNA	P123	RNA ⁺ act.
ts14	E163K	+	-	-	-
ts16	V275I	+	-	-	-
ts21	C304Y	+	-	-	-
ts19	L416S	+	-	-	-
ts18	P509L	+	+	+	-
ts17	A517T	+	+	+	+
ts7	D522N	-	-	-	+
ts133	N700K	+	+	+	+
ts24	K736S	+	+	+	-
ts24R	Q191K ^(*)	-	-	-	+
SFV ts4	M781T	+	+	+	+

(*) mutation in nsP4

Fig. 7. Localization of mutations in nsP2 and properties of SIN temperature-sensitive mutants of complementation group A1 compared with SFV ts4. Mapping of mutations of ts7, ts18, ts21, ts24 and ts133 was by Hahn *et al.* (1989a, b), and those of ts14, ts16, ts19 by Dé *et al.* (1996). The mutation in the ts24 nsP4 gene predicted a change from Q191 to K (not Q195 as originally reported by Sawicki *et al.*, 1990; see Strauss & Strauss, 1994). Temperature-sensitive growth is expressed as efficiency of plating at 39 °C/ 28 °C being at least 10⁻⁶ (+). Temperature sensitivity of 26S RNA synthesis at 39 °C is expressed as (+). Delayed polyprotein (P123) processing at 39 °C (+). Activation of minus-strand RNA synthesis (RNA⁺ act) at 39 °C in the presence of cycloheximide (+).

not to interfere with virus replication, since revertants of SIN nsP2 mutant ts24 have a non-lethal mutation in nsP4 that is responsible for the reactivation of minus-strand synthesis (mutation at nt 6339 originally reported by Sawicki *et al.*, 1990 changes Q191 to K, not Q195; corrected by Strauss & Strauss, 1994; Fig. 7). Interestingly, the SFV ts4 mutation prevented the full, normal shutoff of minus-strand synthesis also at 28 °C (Fig. 6).

Recent results with SIN mutants and SFV ts4 (this study) have shown that all nsPs participate in minus-strand synthesis: (i) nsP1 as a specific 'initiation factor' (SIN ts11), (ii) nsP3 to form the initial complex (SIN ts4, Sawicki *et al.*, 1981b, 1990; LaStarza *et al.*, 1994; Wang *et al.*, 1994) and (iii) nsP2 and nsP4 are involved in the synthesis and shutoff of minus-strand

transferred to 39 °C at 6 h p.i. and incubation continued for 2 h (lanes 3, 4, 7, 8, 11, 12, 15 and 16). At 8 h p.i. cells from 28 °C and 39 °C incubations were harvested, lysed and the postnuclear supernatant fractionated to yield P15 (P) and S15 (S). Equivalent amounts of resuspended P15 and S15 fractions were subjected to immunoprecipitation with antisera against nsP1 (lanes 1–4), nsP2 (lanes 5–8), nsP3 (lanes 9–12) and nsP4 (lanes 13–16) after solubilization with 1% SDS. The precipitates were analysed by SDS-PAGE in 7.5% acrylamide gels followed by fluorography. (b) ts4-infected cultures labelled as in (a) were transferred first for 60 min to 39 °C (lanes 1, 2) followed by shift back to 28 °C for 60 min (lanes 3, 4). After isolation of P15 and S15 fractions the proteins were analysed by immunoprecipitation with anti-nsP2 antiserum alone. (c) BHK cells infected and labelled as in (a) were fractionated into P15 and S15 followed by exposure to reversible cross-linking agent DTSP as described in Methods, and immunoprecipitation with anti-nsP2 antiserum as in (b). SDS-PAGE analysis of the precipitates from ts4- and wt-infected cells incubated at 28 °C and 39 °C was carried out in 15% gels.

synthesis (Fig. 7; this study; Sawicki & Sawicki, 1993; Wang *et al.*, 1994). Evidently very subtle changes control the template specificity of the RNA polymerase complex, since stable polymerase can regain minus-strand synthesizing capacity from point mutations in either nsP2 or nsP4, indicating rearrangement of cleaved nsPs to the same configuration as in the initial and unstable RNA polymerase complex (P123 + nsP4 or nsP1 + P23 + nsP4; Lemm *et al.*, 1994; Shirako & Strauss, 1994; Wang *et al.*, 1994). The change is induced by shift to 39 °C (SIN ts17, ts133 ts24R1 and R2 revertants; Sawicki *et al.*, 1990; Sawicki & Sawicki, 1993) or it may be constitutive as in SFV ts4 (this study).

Alphavirus nsP2 has several different functions in RNA replication and transcription. Some of these functions (e.g. autoprotease, NTPase, RNA helicase?) are carried out in close contact with the other nsPs. However, for the regulation of 26S RNA synthesis a separate soluble nsP2 is evidently required. We assume that this form of nsP2 recognizes the 26S RNA promoter acting as an initiation factor for 26S RNA synthesis. As a regulator it could have an RNA binding site, which might well be at or close to the carboxy terminus where the 26S RNA inhibitory mutations are clustered (Fig. 7). It should also have an activator domain for the recognition of the core polymerase complex. Search for RNA-binding motifs among RNA-binding proteins did not reveal any previously identified amino acid sequence motifs in nsP2 (Birney *et al.*, 1993). It is equally possible that as a regulator nsP2 operates by binding to another protein, which is in direct contact with the 26S RNA promoter.

The ability of nsP2 to migrate alone to the nucleus (Rikkonen *et al.*, 1992) and to associate with ribosomes in the cytoplasm (Ranki *et al.*, 1979) indicates free mobility of a part of the nsP2 population. Here we suggest that nsP2 has two different roles in RNA replication and transcription. It is part of the RNA polymerase core, but in addition it serves as a soluble regulatory component in the transcription of 26S RNA. Also the protease function of nsP2 is accomplished in two different ways. During the translation of the P1234 polyprotein nsP2 cleaves the precursor *in cis* but later in infection cleavage *in trans* enhances the polyprotein processing significantly, resulting in the shutoff of minus-strand RNA synthesis (Strauss & Strauss, 1994). Thus, nsP2 exercises its functions in a mobile form and as a component of the RNA polymerase complex.

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